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HYDROXAMIC ACID CONTENT AND TOXICITY OF RYE AT SELECTED GROWTH STAGES

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Abstract—Rye (*Secale cereale* L.) is an important cover crop that provides many benefits to cropping systems including weed and pest suppression resulting from allelopathic substances. Hydroxamic acids have been identified as allelopathic compounds in rye. This research was conducted to improve the methodology for quantifying hydroxamic acids and to determine the relationship between hydroxamic acid content and phytotoxicity of extracts of rye root and shoot tissue harvested at selected growth stages. Detection limits for an LC/MS-MS method for analysis of hydroxamic acids from crude aqueous extracts were better than have been reported previously. (2*R*)-2-*β*-D-Glucopyranosyloxy-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA-G), 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA), benzoxazolin-2(*3H*)-one (BOA), and the methoxy-substituted form of these compounds, (2*R*)-2-*β*-D-glucopyranosyloxy-4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA glucose), 2,4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA), and 6-methoxy-benzoxazolin-2(3*H*)-one (MBOA), were all detected in rye tissue. DIBOA and BOA were prevalent in shoot tissue, whereas the methoxy-substituted compounds, DIMBOA glucose and MBOA, were prevalent in root tissue. Total hydroxamic acid concentration in rye tissue generally declined with age. Aqueous crude extracts of rye shoot tissue were more toxic than extracts of root tissue to lettuce (*Lactuca sativa* L.) and tomato (*Lycopersicon esculentum* Mill.) root length. Extracts of rye seedlings (Feekes growth stage 2) were most phytotoxic, but there was no pattern to the phytotoxicity of extracts of rye sampled at growth stages 4 to 10.5.4, and no correlation of hydroxamic acid content and phytotoxicity (I_{50} values). Analysis of doseŸresponse model slope coefficients indicated a lack

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of parallelism among models for rye extracts from different growth stages, suggesting that phytotoxicity may be attributed to compounds with different modes of action at different stages. Hydroxamic acids may account for the phytoxicity of extracts derived from rye at early growth stages, but other compounds are probably responsible in later growth stages.

Key Words V DIBOA glucose, (2*R*)-2-*β*-D-glucopyranosyloxy-4-hydroxy-(2*H*)-1, 4-benzoxazin-3(4*H*)-one, DIBOA, 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one, BOA, benzoxazolin-2(*3H*)-one, DIMBOA glucose, (2*R*)-2-*β*-D-glucopyranosyloxy-4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one, DIMBOA, 2,4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one, MBOA, 6-methoxy-benzoxazolin-2(3*H*)-one.

INTRODUCTION

Rye (*Secale cereale* L.) is a popular winter annual cover crop in conventional and conservation tillage production systems of major vegetable and field crops including sweet corn (*Zea mays* L.) (Burgos and Talbert, 1994), soybean (*Glycine max* L.) (Liebel et al., 1992), and tomatoes (*Lycopersicon esculentum* Mill.) (Masiunas et al., 1995). Rye has many desirable attributes that make it a suitable winter cover crop for cool, northern climates: seeds germinate quickly, seedlings establish in cool weather, young plants produce deep roots that reduce soil erosion and recycle nutrients, and mature plants yield 7Y8 t/ha of biomass that can enhance soil tilth. Rye residues exhibit numerous physical and chemical attributes that can influence subsequent crops and their environment. Physical attributes of rye on the surface of soils can affect the radiation, thermal, and hydrological environment that in turn can influence emerging crops, weeds, and pests (Teasdale and Mohler, 1993; Teasdale et al., 2004). Chemical release of toxins and production of phytotoxic microbial products from rye residues has been widely documented (Putnam, 1985; Niemeyer, 1988; Weston, 1996).

Allelopathy in rye is attributed to two major compounds: 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA) and its breakdown product, benzoxazolin-2(*3H*)-one (BOA) (Barnes et al., 1987). Both DIBOA and BOA are abundant in rye. They arise sequentially as DIBOA glucose (DIBOA-G) is enzymatically degraded to DIBOA. BOA is formed by the natural breakdown of the DIBOA (Niemeyer, 1988). Additional allelopathic compounds have also been identified in rye (Shilling et al., 1986), but they are considered to have lower activity and are seldom considered when chemical-specific allelopathy studies on rye are carried out (Barnes et al., 1987; Niemeyer, 1988).

The concentrations of DIBOA and BOA in rye may be affected by many variables. Burgos et al. (1999) reported over a tenfold difference in the concentration of DIBOA among eight cultivars. Mwaja et al. (1995) showed that DIBOA and BOA concentrations and allelopathic activity varied with plant

fertility, being higher when rye was grown at low to moderate fertility than at high fertility. Reberg-Horton et al. (2005) found major concentration differences of DIBOA within several rye cultivars based on plant age and stage of development. Several studies have compared differences in phytotoxicity of rye root *versus* shoot tissue with conflicting results (Barnes and Putnam, 1986; Chase et al., 1991; Hoffman et al., 1996). Research is needed to determine changes in allelopathic compounds in rye tissue as a function of plant development.

When assessing the main allelopathic chemicals in rye, many researchers have attributed the major activity to DIBOA (Barnes et al., 1987; Burgos and Talbert, 2000). In the intact rye plant, the major form of DIBOA is the glycone form, DIBOA glucose (DIBOA-G), which rapidly loses the glucose moiety through enzymatic cleavage when the plant tissues are damaged (Hietala and Virtanen, 1960). Subsequent breakdown of DIBOA to BOA is enhanced through heating and mechanical action of the macerated plant extracts (Tang et al., 1975). These three DIBOA-related compounds make up the hydroxamic acid content in rye as described by Burgos et al. (1999). However, the methoxy-substituted family of hydroxamic acids has also been detected in rye, e.g., DIMBOA glucose, DIMBOA (Hofman and Hofmanová, 1969) and MBOA (Tang et al., 1975). Thus, to assess the total hydroxamic content of rye, one needs to measure both the DIBOA family, referred to here as the hydroxamic acid (HA) content, and the DIMBOA family, referred to as methoxy-substituted (MHA). Note that HA also has been classified as "BX^ by Reberg-Horton et al. (2005) in reference to the fact that "B^ and "X^ are unique letters in the chemical name common to all of these compounds, "benzoxaz...one.^ Assessment of the total hydroxamic acid content of rye (HA + MHA) was a major goal of this study.

There are few analytical methods that have adequately accounted for all members of the hydroxamic acid (HA + MHA) family of compounds from rye. Early research focused on methods utilizing colorimetry and spectrophotometry by measuring the absorbance of a blue complex with $FeCl_3$ (Baker and Smith, 1977). This approach was laborious and limited by the fact that benzoxazolinones (BOA and MBOA) do not react with $FeCl_3$ (Lyons et al., 1988). At present, identification and quantification of HA and MHA are typically done by high performance liquid chromatography-ultraviolet (HPLC-UV) (Gutierrez et al., 1982; Lyons et al., 1988; Burgos et al., 1999) or gas chromatography (GC) (Tang et al., 1975; Woodward et al., 1979a,b). However, none of these methods is capable of detecting low (<1 μg/g dry wt) concentrations in rye. Gas chromatographic (GC) methods, both GC/flame ionization (FID) (Tang et al., 1975; Woodward et al., 1979a) and GC/mass spectrometry (MS) (Woodward et al., 1979b), provide sensitivities similar to HPLC-UV methods, and the MS method provides additional qualitative assurance of compound identity. However, current GC methods require lengthy derivatization steps that make the techniques

more complicated than LC. Only two studies report the presence of MHA components in rye, Hofman and Hofmanová (1969) and Tang et al. (1975). Most investigators do not look for the MHA compounds, even though Tang et al. (1975) reported fairly high levels in their study, i.e., 120 μg/g fresh wt. leaves of rye and 310 μg/g fresh wt. in rye roots using gas chromatographic methods. They reported 710 μg/g fresh wt. of BOA in these same extracts.

With the advent of liquid chromatography coupled to triple quadrupole mass spectrometry using electrospray ionization methods (LC/ESI/MS-MS), and the numerous improvements to simultaneous qualitative and quantitative characterization of liquid chromatographically separable materials that it offers, we decided to use these methods to develop a new analytical method for determining the analysis of benzoxazinone derivatives in rye extracts. Other investigators (Cambier et al., 2000; Bonnington et al., 2003a) explored variations of these methods for analysis of benzoxazinones in wheat and corn. The analytical goals were to develop an LC/ESI/MS-MS method that would work directly on crude aqueous extracts and one that would provide lower method detection limit values than currently available. Consequently, this study was undertaken to: (1) develop an LC/ESI/MS-MS method to work directly on crude aqueous extracts and lower detection limits; (2) evaluate phytotoxic activity in aerial and root tissues at various stages of plant growth from seedling to maturity; and (3) determine potential correlations between hydroxamic acid concentration and phytotoxicity at these different growth stages.

METHODS AND MATERIALS

Plant and Tissue Preparation. The experiments were conducted at the Beltsville Agricultural Research Center, Maryland, USA. Common "Abruzzi^ rye was seeded in mid-September, 2002, using a no-tillage John Deere grain drill (Model No. 450, Westminster, MD, USA). Seeding rate was 90 kg/ha in rows 19 cm apart. No herbicide, fertilizer, or irrigation was applied until the last sample was taken on 17 June 2003. Rye aerial and root tissue was sampled at five dates from March through June of 2003, covering growth stages 4 to 10.5.4 on Feekes growth scale (Large, 1954), and again in November of 2002 corresponding with growth stage 2. Only aerial tissue was taken on the June sampling date due to the difficulty of recovering senescing root tissues.

Whole plants with intact roots were carefully dug, and the soil was washed gently from the roots. Weeds were separated and discarded. Aerial tissues were separated from roots, and the tissues were washed several times with distilled water and blotted on paper. They were then cut into 5-cm pieces, dried for 3 d in a forced-air oven at 65°C, ground in a Wiley mill (Model No. A75-A, Arthur Thomas Co., Philadelphia, PA, USA) to pass through a 40-mesh screen, and

stored in airtight bags at 4°C until use. Aqueous crude extracts from aerial and root ground tissues were prepared following established procedures (Barnes and Putnam, 1986; Burgos et al., 1999; Burgos and Talbert, 2000) with slight modification. Ground tissues (12 g) were incubated in 180 ml deionized water (1:15 w/v) in 500-ml Erlenmeyer flasks. The flasks were sealed with a parafilm layer, placed on a shaker (Gyrotory G-2; Edison, NJ, USA), and incubated for 24 hr in the dark at 4°C with constant shaking at 100 rpm. The slurry was filtered through six layers of cheesecloth and centrifuged for 10 min at 3,046 $\times$ *g* in a refrigerated centrifuge. The supernatant solution (134 $\pm$ 2 ml), which represented the crude aqueous extract, was held in ice until used in bioassays. Electrical conductivity of the crude extract was determined using a conductivity meter. Portions of the supernatant were filtered by using a 0.7-μm Whatman glass microfiber filter, and aqueous filtrate solutions were injected directly onto an LC column connected to the LC/ESI/MS-MS as will be described later.

Analytical Procedures. Chemicals used were derived from the following sources: benzoxazolin-2(3*H*)-one (BOA) (98%) and 6-methoxy-benzoxazolin-2(3*H*)-one (MBOA) (97%) were bought commercially from Sigma-Aldrich (St. Louis, MO, USA); 2,4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA) and (2*R*)-2-*β*-D-glucopyranosyloxy-4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA-G; estimated >90%) were isolated from corn according to the methods described by Klun et al. (1967); and 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA) and (2*R*)-2-*β*-D-glucopyranosyloxy-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA-G) were synthesized following the methods of Sicker et al. (1989) for DIBOA (estimated >95%) and Kluge et al. (1997) for DIBOA-G (estimated >95%).

Analytical method development was aimed at improving the detection limits for the hydroxamic acids expected to be present in the various extracts. Initial full-scan analyses were carried out to determine what types of hydroxamic acid-related compounds could be found. The initial data produced spectra that could potentially arise from the hydroxamic acid family of compounds. Therefore, standards were obtained or synthesized to quantify these compounds as well as β-hydroxybutyric acid and β-phenylacetic acid.

Spike recovery experiments were carried out on leaf extracts from rye sampled at Feekes stage 2. These extracts produced the greatest amount of co-extracted materials, i.e., pigments and suspended cell materials that tended to clog the 1-μm pore size syringe filters used to clarify extracts for LC injection. Because of this clogging, only about 1 ml of material could be obtained before clogging occurred.

Instrumentation. The LC instrument was a Waters 2690 XE Separation module (Waters Corp., Milford, MA, USA). The LC column was a Waters X-Terra MS C-18 column, (5 μm 2.1 $\times$ 150 mm). The separation method was

adapted from Lyons et al. (1988). These conditions were set using known standards of the analytes. The liquid chromatographic protocol involved an isocratic elution program after a 10-μl injection of the sample. The mobile phase consisted of 75% solvent A, 30:70 (v/v) mixture of methanol and 1% formic acid and 25% solvent B, distilled water. A flow rate of 0.3 ml/min was maintained while maintaining the column temperature at 45°C.

Electrospray ionization mass spectrometry (positive and negative ion; ES+/−, MS/MS) was performed on a Quattro LC benchtop triple quadrupole mass spectrometer (Micromass Ltd., Manchester, UK) and analyzed using the multiple reaction monitoring mode (MRM). Exact identification and quantitation was aided by the specificity of the MS that monitored characteristic daughter ions from parent masses of BOA, DIBOA, DIBOA-G, MBOA, DIMBOA, and DIMBOA-G, and the selectivity of the LC separation producing matching peaks at the predetermined retention times for the standards. Nonspecific mass spectrometer settings were set as follows: capillary voltage was set at 3.0 kV; source and desolvation temperatures were set at 140 and 400°C; liquid nitrogen was used to supply the nebulizer and desolvation gas (flow rates were approximately 70 and 600 l/h, respectively). Argon was used as collision-induced decomposition gas to fragment the parent ions, typical pressure 2.9×10^{-5} mbar. MRM was chosen because it allows high sensitivity and selectivity by setting both quadrupoles to transmit selected ions only; the goal of analysts is typically to set the first quadrupole to select the quasi-molecular ($[M\text{-}H]^+$ or $[M\text{-}H]^-$) molecular weight ions (the exceptions here were DIBOA and DIMBOA-G; Table 1) that are allowed to react in the collision cell, and the second quadrupole is set to transmit only specific daughter ions. The compound-specific settings for each parent and daughter ion used for compound identification and quantification are listed in Table 1, along with the optimized cone voltages and collision energies used. Resulting retention times for the specific column conditions are also shown.

TABLE 1. COMPOUND-SPECIFIC MASS SPECTROMETER SETTINGS AND LC RETENTION TIMES

Compound	Parent ion (m/z)	Daughter ion (m/z)	Ionization mode	Retention time (min)	Cone (V)	Collision (eV)	Molecular weight
DIMBOA-G	380	218	ES+	5.8	36	20	373
DIMBOA	212	166	ES+	7.7	12	9	211
MBOA	166	110	ES+	11.4	35	20	165
DIBOA-G	342	134	ES−	5.3	15	11	343
DIBOA	164	80	ES+	7.0	15	20	181
BOA	136	108	ES+	10.2	35	16	135

Quantification was carried out by external standard method using a 5-point standard curve that included 0 with standards ranging from 0.05 to 5 ppm. Peak integration and quantification were performed automatically using the MassLynx 4.0 software (Micromass Ltd.) Instrumental Limit of Detection (LOD) and Limit of Quantification (LOQ) were estimated in terms of the baseline noise. LOD values were calculated using the Eurochem guidelines, where the standard deviation of the lowest visible standards was multiplied by 3.3 to provide method detection limit values (Standards Council of Canada, 2003). Quantification limits were based on the lowest standard that produced a signal to noise ratio greater than 3:1 (Table 2).

Bioassay Methods. Crude aqueous extracts were prepared in proportions of 1.0, 0.5, 0.25, and 0.125 for use in Petri dish bioassays to determine the suppressive activity of rye aerial and root tissue on germination and growth of "Great Lakes^ lettuce and "Rutgers^ tomato seeds. Lettuce and tomato seeds (50 per dish) were germinated in 100 × 15 mm Petri dishes over a Whatman No. 1 filter paper using 2.5 ml of extract per dish. Dishes were sealed with parafilm, placed on trays, and held in the dark in a growth chamber at a constant 26°C. Trays were positioned at a 45° angle to encourage geotropic growth and facilitate hypocotyl and root measurements (Burgos and Talbert, 2000). Percent germination and hypocotyl and root lengths were measured after 3 d for lettuce and after 5 d for tomatoes. There were five replications for each sampling date, extract, and proportion, and all extractions were repeated once. Data were expressed as the ratio of the treatment value divided by the control in which seeds were germinated in deionized water.

At the May 27 sampling, rye plants reached a growth stage of 10.3 on the Feekes scale. By that time, flowering was complete and the uppermost leaves were still green, whereas the lower leaves were senesced. Plants were harvested, and the aerial tissues were separated into upper green leaves, lower senesced

TABLE 2. ANALYTICAL METHOD PERFORMANCE

Compound	Limits of quantification (μg/g dry wt.)	Limits of detection (μg/g dry wt.)	Spike recoveries (%)	Precision for spike tests [(RSD[a,b] (replicates)]
DIMBOA-G	0.75	0.4	60	0.18 (N = 6)
DIMBOA	0.84	0.1	104	0.12 (N = 9)
MBOA	0.36	0.7	102	0.07 (N = 9)
DIBOA-G	0.75	0.6	60	0.10 (N = 9)
DIBOA	0.84	0.3	125	0.04 (N = 9)
BOA	0.36	0.5	102	0.03 (N = 9)

[a] RSD = relative standard deviation of the means.
[b] Spike concentrations varied from an average of 39Y120 μg/g dry wt.

leaves, and leafless stems. The tissues were dried and ground as described earlier. Crude extracts from these tissues were used in bioassays to determine suppressive activity.

Statistical Analysis. All analyses were conducted using SAS Version 8.2 (SAS Inc., Cary, NC). Analysis of variance and mean separations were conducted using PROC MIXED. The nonlinear doseYresponse function $Y = 100/(1 + (X/c)^{**}b)$, where Y is the assay species response, X is the extract proportion, and c and b are coefficients, was determined using PROC NLIN (Seefeldt et al., 1995). The I_{50} value was determined by the c coefficient. Correlations among hydroxamic acid concentrations and I_{50} values were determined using PROC CORR.

RESULTS AND DISCUSSION

Analytical Observations. DIBOA and BOA (HA group) were the major compounds measured in shoot extracts, whereas DIMBOA-G and MBOA (MHA group) were the dominant constituents of root extracts (Table 3). The relative proportions of BOA and DIBOA agree with data from rye reported by other investigators (Barnes et al., 1987; Mwaja et al., 1995; Burgos et al., 1999; Reberg-Horton et al., 2005). The proportions depend on how completely the enzyme has reacted to remove the glucose precursor, and this may vary depending on extract preparation methods. Furthermore, many researchers do not monitor all of the precursor species or the methoxy-substituted forms. Our rediscovery of MBOA in rye may have significance as an important chemical responsible for toxicity.

Precision checks during routine processing resulted in duplicate analyses, one leaf and one root sample both from rye sampled at Feekes stage 2. For DIBOA in leaves, the relative standard deviation (RSD) was 0.08 [relative percent difference (RPD) of 12%], for BOA in the leaves the RSD was 0.22 (RPD 30%), and for root it was 0.47 (RPD 67%), and for MBOA in the root sample the RSD value was 0.19 (RPD 27%). Based on these routine tests, the overall precision of the analyses during processing of the samples was considered acceptable, e.g., <0.25, except for BOA in roots, which may have been caused by the relatively low concentration of this compound in these samples. Precision of the general method was also tested by using spike recovery experiments, where triplicate sample sets were analyzed on high (average of 120× background), low (average of 40× background), and nonspiked materials (concentrations ranged from 0.8 to 500 μg/g dry wt). There did not appear to be any effect of concentration on the precision of these results, which allowed all replicates to be averaged. Thus, the respective

TABLE 3. TOTAL HYDROXAMIC ACID (HA + MHA) CONCENTRATION (μg/g dw) IN RYE PARTS HARVESTED ON VARIOUS DATES

	HA			MHA					
Sampling date	DIBOA glucose	DIBOA	BOA	DIMBOA glucose	DIMBOA	MBOA	Total HA	Total MHA	Total All
Rye shoot tissue									
10 Nov.	2.8	287	114	Y	Y	2.7	404	2.7	407
24 Mar.	1.4	108	96.0	Y	Y	2.2	205	2.2	208
15 Apr.	Y	12.8	60.9	Y	Y	2.7	73.7	2.7	76.4
6 May	17.2	15.0	22.8	Y	Y	0.7	55.0	0.7	55.7
27 May	7.7	4.0	3.7	Y	Y	<0.36 (0.32)	15.4	0.3	15.7
17 Jun.	Y	2.3	2.3	Y	Y	Y	4.6	Y	4.6
Rye root tissue									
10 Nov.	2.2	6.6	22.5	Y	1.0	155	31.3	156	187
24 Mar.	Y	0.9	13.8	Y	Y	36.0	14.7	36.0	51.7
15 Apr.	lost	lost	lost	lost	lost	lost			
6 May	7.6	Y	11.4	43.4	1.1	11.9	19.1	56.3	75.3
27 May	10.5	Y	4.0	38.8	Y	7.2	14.5	46.1	61.6

average RSD values for the analytes measured by this method were as follows: 0.18, 0.12, 0.07, 0.10, 0.04, and 0.03 for DIMBOA glucose, DIMBOA, MBOA, DIBOA glucose, DIBOA, and BOA, respectively (Table 2).

Spike recovery tests with Feekes stage 2 material revealed that all compounds were recovered at or greater than 60% (Table 2). The glucose-substituted forms of the hydroxamic acids were consistently low (60% ± 2.1 to 3.6% standard error, respectively, for DIBOA-G or DIMBOA-G compared to 102Y 125% for the other nonglucose-substituted compounds). Because of the low and consistent recovery of the two glucose-substituted forms, there could be a small amount of matrix suppression occurring here. This type of interaction is not uncommon when using the electrospray interface (Reemtsma, 2001). These matrix effects need to be assessed, especially when working with more contaminated extracts, such as were encountered in these analyses. Methods to correct for these effects include standard additions or passing the crude extracts through solid-phase cleanup cartridges, e.g., C-18 cartridges (Bonnington et al., 2003a; Schmitz-Afonso et al., 2003). The ideal method to compensate for possible matrix effects with these methods is to use isotope dilution methods utilizing labeled standards that behave exactly as the unlabeled materials. Even though they coelute with the natural material, each can be separately identified and distinguished because of unique molecular weights. Unfortunately, such isotopes are sparingly available and often require costly and time-consuming custom synthesis.

Whereas most current LC/UV methods require several steps to isolate the hydroxamic acid and related products, i.e., aqueous-phase extractions followed by organic solvent extractions of the aqueous phase and final solvent exchanges and filtration (Gutierrez et al., 1982; Lyons et al., 1988; Yenish et al., 1995; Melanson et al., 1997), our method requires a simple aqueous phase extraction, filtration, and analysis. Niemeyer et al. (1989) successfully employed a similar preparation procedure using LC/UV detection (263 nm) to analyze DIBOA and DIMBOA in wheat and rye tissues. Aside from some graphical data on spike performance, recoveries near 100%, and what appears to be low standard deviation of their replicated spikes, there was little additional detail on method performance. The authors made no mention of detecting any benxozalonones (BOA and MBOA) in their extracts.

Our studies had one drawback, namely, that crude extracts have limited shelf lives and must be analyzed within 1 d of preparation or the HA and MHA compounds appear to degrade. If longer holding times are necessary, then organic solvent extractions could be employed or additional studies might be needed. There appeared to be some minor matrix effects with the most contaminated extracts; however, cleanup of these extracts did not appear to be necessary because accurate quantitative and qualitative determinations were possible.

Detection limits were better than have been reported by others (Table 2), with the exception of the Bonnington et al. (2003a), who also employed LC/ESI/MS-MS methods. Their limit of detection values (μg/g dry wt) varied from 0.1 to 1.1, whereas our values ranged from 0.1 to 0.7 for the same analytes. Lyons et al. (1988) reported detection limits with their method, HPLC with UV detection (λ = 280 nm), in terms of lowest standard injected for DIMBOA-G and DIMBOA of 0.2 nmol (2.2 ppm DIMBOA and 3.8 ppm DIMBOA-G). Comparing this to our lowest standard of 0.056 ppm that was injected in 10 μl, our detection range was >39 times and >140 times lower for DIMBOA and DIMBOA-G, respectively. Using the LOD values in Table 2, our detection limits were >314 times lower than those of Lyons et al. (1988). Using their method for calculating limits of detection, our values computed to range from 0.7 to 2.8 pmol. Woodward et al. (1979a) used a GC/flame ionization detection (FID) method for DIMBOA and DIBOA and reported detection limits for 10 μl injections of 0.02 and 0.05 nmol for DIBOA and DIMBOA, respectively. Their analytical variation was expressed as a standard error of 2% of the mean, and their recoveries were 106% and 78% for DIMBOA-G and DIMBOA, respectively. Their standard error values for precision on five replicates were 434 ± 13 and 1,066 ± 19 for DIMBOA-G and DIMBOA, respectively, in corn that would correspond to RSD values of 0.067 and 0.040, respectively, for DIMBOA-G and DIMBOA. Gutierrez et al. (1982) also used HPLC-UV for quantification of DIMBOA and MBOA in corn extracts and reported recoveries of 104 ± 2% for DIMBOA and 106 ± 3% for MBOA. The variability values were not clearly defined and they did not provide detection limit data.

Qualitative identification of the different analytes is vastly improved using LC/MS methods *versus* non-MS methods discussed above, e.g., LC/UV and GC/FID. Cambier et al. (2000) utilized LC/MS for qualitative and semi-quantative analysis of the glucosylated forms of DIMBOA in corn tissues. They chose to use atmospheric pressure chemical ionization methods (APCI) rather than the electrospray ionization methods utilized here. Their investigation did demonstrate the utility of LC/MS for better characterizing the family of compounds comprising the benzoxazinone group. Bonnington et al. (2003a) conducted a more detailed study using LC/MS. Their LC/ESI/MS-MS methods differed from ours in that they conducted their acquisition in negative ionization mode *versus* the positive electrospray ionization method used here. Under negative ionization methods, the analytes produce different characteristic ions than in positive mode. For their method, more fragment ions were produced and quasi-molecular ions near the molecular weights were seldom detected. This apparently limited the choices of ion transition pairs that Bonnington et al. (2003a) selected for their quantitation parameters. Thus, they used identical daughter ions for three analyte pairs and, in two cases, the parent-to-daughter transition ion pairs were the same for two different compounds (DIMBOA and

MBOA). All of this limited the selectivity of their method, especially when compared to ours where none of the transition pairs were the same and in most cases (4 out of 6) the parent ion was the quasi-molecular $[M-H]^+$or $[M-H]^-$ form for the compound's molecular weight (Table 1). DIBOA was one of the compounds whose quasi-molecular parent ion was different, e.g., 18 mass units lower than its molecular weight; this suggests an initial loss of water from the molecule prior to formation of the MH^+ parent. The daughter mass for this pair was 80 *m*/*z*, which was also identified by Bonnington et al. (2003b). These authors also described many of the other transition ions observed here, excluding the glucosylated compounds. The DIMBOA glucose parent ion had a mass 7 units higher than its molecular weight. A possible explanation for this would be the formation of a sodium adduct of the glycosylated DIMBOA after loss of oxygen. While most sodium adduct molecules are stable to further breakdown, it has been observed that some sodium adducts of glucose-substituted molecules are susceptible to loss of the aglycones under collision induced fragmentation (Tolonen, 2003). Loss of the aglycone (162 *m*/*z*) seems the best explanation for the 282 *m*/*z* daughter fragment observed here, Table 1.

Two additional allelopathic compounds of lower activity, *β*-phenylactic acid and *β*-hydroxybutyric acid (Shilling et al., 1986), were also searched for in the rye extracts. However, consistent levels were not detected and the data are not presented.of these compounde mye

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preparations as it was in extracts of younger tissue. Paired comparisons of extraction results using our aqueous extraction methods *versus* use of organic solvents as has been utilized by other researchers (Lyons et al., 1988; Burgos et al., 1999) might have permitted more HA and MHA compounds to be recovered; however, measuring the levels in the aqueous extracts, as we have done here, was more relevant to the bioassay exposures that was the primary intent of the method.

Some of the important advantages in using our method include: the ease and speed at producing extracts for analysis, e.g., aqueous extraction, followed by filtration and the low detection limits possible, e.g., at least an order of magnitude lower than conventional detection methods, and the certainty of compound identification, since molecular mass identification is part of the detection method. While the detection levels are much lower than may be currently needed to associate concentration with allelopathic activities, following the fate of these compounds in the environment and in their biogenesis in plant tissues clearly becomes easier with these lower detection limits.

Bioassay Observations. Lettuce root growth was the most sensitive assay for phytotoxicity studies. Averaged over all factors, lettuce root length was inhibited by 49% by rye extracts, tomato root length was inhibited by 19%, and none of the other assays using hypocotyl length or germination showed inhibition greater than 10%. Averaged over all factors for both species, hypocotyl length was inhibited by 9%, and germination was the least sensitive assay of all, being inhibited by 4%. These results confirm previous results showing that lettuce root growth is a sensitive indicator of phytotoxic activity (Barnes and Putnam, 1986; Burgos and Talbert, 2000). Our results also confirm previous research showing that root growth is more sensitive than hypocotyl growth (Burgos and Talbert, 2000). Subsequent discussion of our results will focus on the lettuce and tomato root length assays. Similar results were obtained using other assays, but were less pronounced because of the relative insensitivity of these assays.

Across all proportions, extracts from rye shoot tissue were more phytotoxic than extracts from rye root tissue using either the lettuce or tomato root length assays (Figure 1). Barnes and Putnam (1986) also showed that rye shoots were more phytotoxic than rye roots. Lettuce was more sensitive than tomato to both rye shoot and root extracts. Tomato responses to extracts were insufficient to adequately fit the doseYresponse model and determine an I_{50} value; consequently, subsequent analyses of the influence of sampling date will be presented for the lettuce assay only.

Extracts of rye root and shoot tissue that were sampled at selected dates in the spring and fall differed in their phytotoxicity to lettuce root length (Figure 2). The doseYresponse model c parameter represents the proportion of

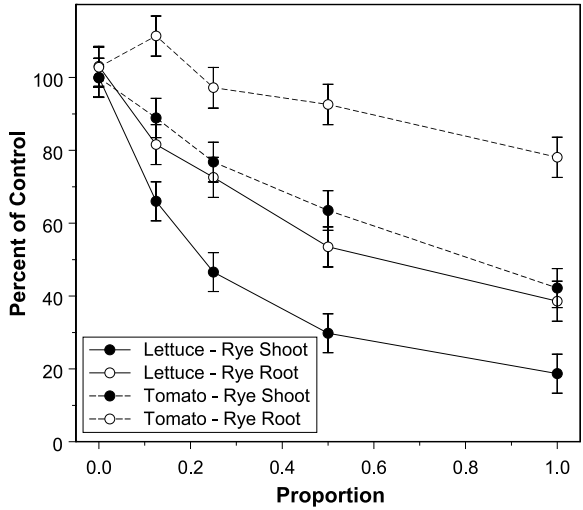

FIG. 1. Lettuce and tomato root length in response to proportions of full-strength extracts of rye shoot or root tissue averaged over spring sampling dates. Error bars represent the standard error (SE) of the mean.

full-strength extract required to inhibit lettuce root length by 50% (I_{50}). The I_{50} values were lowest (indicating the highest level of phytotoxicity) for both rye shoot and root tissue harvested in November, followed by that harvested in April (Table 4). There was not a clear decline in phytotoxicity with age of tissue

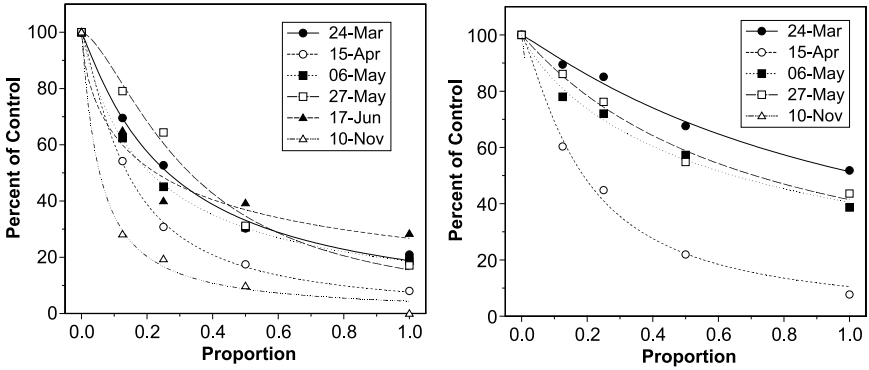

TABLE 4. DOSEYRESPONSE MODELS[a] OF LETTUCE ROOT LENGTH IN RESPONSE TO RYE SHOOT AND ROOT EXTRACTS

Rye tissue	Date	c (I_{50})	b	R^2
Shoot	10 Nov.	0.055 d	1.068 abc	0.78
	24 Mar.	0.264 b	1.100 b	0.82
	15 Apr.	0.140 c	1.267 ab	0.91
	6 May	0.210 b	0.944 bc	0.81
	27 May	0.330 a	1.531 a	0.90
	17 Jun.	0.227 b	0.679 c	0.58
Root	10 Nov.	0.134 d	0.895 b	0.88
	24 Mar.	1.053 a	1.084 ab	0.59
	15 Apr.	0.188 c	1.280 a	0.91
	6 May	0.645 b	0.880 b	0.82
	27 May	0.710 ab	1.017 ab	0.86
	17 Jun.	Y	Y	Y

[a] Parameters c and b are from the model $Y = 100/(1 + (X/c)^b)$, where Y is percent of control and X is proportion of extract. Parameter c represents the proportion of the full-strength extract required to inhibit lettuce root length by 50 percent (I_{50}). All regression models were significant ($P < 0.0001$). Parameters within columns and rye tissue followed by the same letter were not significantly different according to 95% confidence limits.

during the spring months because shoot tissue sampled on March 24, May 6, and June 17 had similar I_{50} values, and root tissue sampled on May 27 had a similar I_{50} value to that sampled on March 24 and May 6.

The model b parameter defines the steepness of slope around the c parameter. A comparison was conducted between the model fitted with a common b parameter for all dates *versus* a model with separate b parameters for each date (Seefeldt et al., 1995). F values for these comparisons were significant for rye shoot extracts ($F = 8.31$, $P < 0.01$) and for rye root extracts ($F = 2.77$, $P < 0.05$), indicating that separate b parameters were required for each date. Comparison of 95% confidence intervals also indicated that b parameters differed between sampling dates (Table 2). The lack of equivalence among these steepness parameters indicates a lack of parallelism among models that suggests different modes of action may have occurred among extracts derived from different aged rye tissue (Streibig et al., 1993). The models also indicate that the b parameters did not follow any clear pattern relative to the age of tissue (Table 2). This result suggests that different compounds were probably responsible for the toxicity of extracts from different aged tissue.

Extracts of rye upper leaf tissue harvested on May 27 were more phytotoxic to lettuce and tomato root length than extracts of lower leaf or stem tissue at extract proportions of 0.5Y1.0 (Figure 3). Extracts of lower leaf tissue, which was senescing at the time of harvest, varied in toxicity, exhibiting activity

rcent of control)
100
80
60
0.8
1.0

greater than that of stem tissue in some cases, but not in others. These data suggest that more actively growing tissue tended to have the more phytotoxic extracts.

Total hydroxamic acid, DIBOA, and BOA as well as electrical conductivity were highest in rye shoot tissue at the earliest growth stage in November and declined as tissue aged through the spring (Figure 4). All of these measurements were highly correlated. Correlation coefficients between BOA and DIBOA were 0.84; between BOA and DIBOA with total hydroxamic acids they were 0.91 and 0.99, respectively; and between BOA, DIBOA, and total hydroxamic acids with electrical conductivity they were 0.86, 0.96, and 0.97, respectively. These high correlations suggest that it would be difficult to determine which hydroxamic acids may have contributed to the phytoxicity of these extracts, or whether other ionic compounds that contributed to electrical conductivity may also have been involved. The phytotoxicity of extracts did not follow a similar steady decline over time (Table 2) as that of the compounds in Figure 4. Correlations between phytoxicity as determined by I_{50} values and either the prominent hydroxamic acids, their totals, or electrical conductivity were not significant for rye shoot or root tissue. Burgos et al. (1999) also reported a low correlation between phytoxicity and HA content. These results suggest that compounds other than hydroxamic acids contributed to phytoxicity. DIBOA has been shown to be more phytotoxic than BOA (Barnes et al., 1987; Burgos and Talbert, 2000) and to be capable of inhibiting lettuce root growth at the concentrations observed in our November and March extracts (Burgos and Talbert, 2000). Thus, hydroxamic acids probably contributed to the phytoxicity of tissue sampled at early growth stages, but had minimal contribution at later harvest dates when tissue concentrations dropped to low levels.

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